

# Biomarkers of Cervical Carcinogenesis Associated with Genital Human Papillomavirus Infection



## Indicadores de Prognóstico da Carcinogénese do Colo do Útero Associada à Infeção por Vírus do Papiloma Humano

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### ABSTRACT

**Introduction/Objective:** Persistent infection with high-risk human papillomavirus (HPV) types is a necessary cause for cervical cancer development. The aim of this study was to evaluate the significance of different molecular markers for cervical carcinogenesis, and to assess their association with cervical intraepithelial neoplasia.

**Materials and Methods:** 378 cervical samples from women attending to primary Health Clinics of the National Health Service and Gynaecological Outpatient Clinics and referred for HPV testing were analyzed between January 2007 and December 2010. According to cytological diagnosis, five groups were defined: normal, ASCUS, LSIL, HSIL, and ICC. For the determination of viral DNA physical status was performed by using a real-time PCR methodology, over expression of E6/E7 mRNA NASBA amplification was performed with the NucliSENS EasyQ HPV assay and viral load was determined by a real-time PCR. HPV status was studied in relation to lesion severity. Statistical analysis was performed with SPSS software 16.0 and Chi-Square test.

**Results:** No significant statistical differences were found between the physical status of HPV 16 or 18 and lesion severity. Over-expression of E6/E7 mRNA increased with lesion severity. Viral load was significantly associated with the development of cervical intraepithelial lesion.

**Conclusions:** Data suggests that viral integration for HPV 16 seems to be an early event on cervical carcinogenesis, not being suitable as a molecular marker. E6/E7 mRNA and viral load can be more valuable approaches to use as biomarkers in the prevention of cervical cancer development.

**Keywords:** Cervical Intraepithelial Neoplasia; Papillomavirus Infections; Uterine Cervical Neoplasms; Tumor Markers, Biologica.

### RESUMO

**Introdução/Objetivos:** A infeção persistente pelo Vírus do Papiloma Humano de alto risco (HPV<sub>ar</sub>) é considerada como a causa necessária, embora não suficiente, para o desenvolvimento do cancro do colo do útero, sugerindo que outros fatores estarão envolvidos no processo de carcinogénese. Este estudo pretendeu avaliar indicadores de prognóstico da persistência da infeção por HPV, nomeadamente o estado físico e a carga viral dos HPV 16 e 18 e a superexpressão dos transcritos do RNAm dos HPV 16, 18, 31, 33 e 45, num grupo de mulheres com ou sem sintomatologia clínica e citopatológica.

**Material e Métodos:** Foram estudadas 378 alíquotas de células epiteliais congeladas pertencentes a utentes dos centros de saúde do Serviço Nacional de Saúde e de clínicas privadas, referenciadas para teste HPV, entre Janeiro de 2007 e Dezembro de 2010. De acordo com o diagnóstico citopatológico, foram definidos cinco grupos: normal, ASCUS, LSIL, HSIL e carcinoma invasivo do colo do útero. Para a determinação do estado físico do DNA e da carga viral dos HPV 16 e 18 foi utilizada metodologia de PCR em tempo real, e para a superexpressão dos transcritos dos oncogenes E6 e E7 o sistema comercial NucliSENS EasyQ HPV<sup>®</sup>. Os indicadores foram analisados em associação com os tipos de lesão do colo do útero. Para a análise estatística foi utilizado o programa informático SPSS versão 16.0 e o teste de Chi-Quadrado.

**Resultados:** Os resultados mostraram ausência de associação estatisticamente significativa entre a gravidade da lesão e o estado físico do DNA dos HPV 16 e 18. A superexpressão dos transcritos do RNAm E6/E7 e a carga viral dos HPV 16 e 18 aumentaram significativamente em função do grau da lesão.

**Conclusões:** Os resultados obtidos sugerem que a determinação do estado físico do DNA dos HPV 16 e 18, isoladamente, não constitui um indicador de prognóstico para o desenvolvimento e progressão das lesões. A superexpressão dos transcritos dos oncogenes E6 e E7 está associada à progressão das lesões do colo do útero e apresenta maior especificidade no diagnóstico precoce das lesões pré-malignas. A quantificação do DNA dos HPV<sub>ar</sub> pode ser um indicador promissor de prognóstico das lesões pré-neoplásicas do colo do útero.

**Palavras-chave:** Infeções por Papillomavirus; Marcadores Tumoraes; Neoplasia Intraepiteliais Cervical; Neoplasias do Colo do Útero.

### INTRODUCTION

Cervical cancer is the third most frequent female neoplasm in the world.<sup>1</sup> In Portugal, its incidence is of 12.2 and mortality rate is of 3.6 per 100,000.<sup>1</sup> Genital infection caused by Human Papillomavirus (HPV) is one of the most frequent sexually transmitted diseases (STD) and estimates suggest a prevalence of 30-60% and a higher incidence in younger age groups.<sup>2</sup> Cervical HPV infection presents

several clinical manifestations, from benign lesions to invasive carcinomas.<sup>3</sup> In fact, about 99.7% of the cases of cervical cancer are related with this viral infection.<sup>3</sup> Although most of HPV infections are subclinic and transient, a small percentage of cases progress to an invasive cervical carcinoma for which the HPV type is the most important determining factor.

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There are currently more than 120 HPV types identified, 45 of which infect the genital mucosa. According to epidemiological and phylogenetic studies and based on their oncogenic potential, genital HPV may be classified as high-risk (hr-HPV: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59), possible or probable high-risk (HPV 26, 53, 66, 67, 68, 70, 73 and 82), low-risk (lr-HPV: 6, 11, 13, 40, 42, 43, 44, 54, 61, 72, 81 and 89) and undetermined risk (HPV 2, 3, 7, 10, 27, 28, 29, 30, 32, 34, 55, 62, 69, 71, 74, 77, 83, 84, 85, 86, 87, 90 and 91).<sup>4,5</sup>

A persistent HPV infection is necessary, although not sufficient, for cervical cancer development, suggesting that other factors are involved in carcinogenesis.

The hr-HPV E2 gene disruption leads to a linearisation of the viral genome and over-expression of oncogenes E6 and E7 transcripts (E6/E7 mRNA). When in excess, the oncoproteins E6 and E7 interact with cellular proteins p53 and pRB and promote genomic instability of the infected cells.<sup>6,7</sup> At the same time, DNA integration in the host cell genome is considered a pre-requisite for carcinogenesis<sup>8</sup>. Therefore, the identification of DNA physical status, in an episomal or linear form and the detection of the viral oncogenes E6 and E7 transcript over-expression, in particular from HPV 16, 18, 31, 33 and 45, are important carcinogenesis indicators and provide useful information regarding the risk of progression and/or malignant transformation of the pre-cancerous cervical cells. In fact, the persistence of E6/E7 mRNA over-expression may be one of the major indicators of carcinogenesis.

Several studies have demonstrated that viral load influences predictive value, as well as the association between the viral load increase, the persistence of the infection and lesion grade.<sup>9-11</sup> Low-risk lesions present a 60 times higher risk of progression to a pre-malignant lesion in the case of a high viral load detection.<sup>12</sup> Nevertheless, despite the evaluations made in Europe and in the USA,<sup>13,14</sup> correlation between viral load and lesion severity has still not been fully demonstrated.

This study aimed to assess some prognostic indicators of HPV infection persistence, namely physical status and viral load of HPV 16 and 18 and mRNA transcript over-expression of HPV 16, 18, 31, 33 and 45, in the presence or absence of symptoms and cytopathology.

## MATERIAL AND METHODS

### Population

This study included 378 patients living in Portugal, with ages ranging between 18 - 71 (average = 34.3 ± 10.4; median = 32). These patients had been referred for HPV testing and were randomly selected from Health National Service Health Centres and Private Clinics users, between January 2007 and December 2010. No gynaecological sampling was exclusively performed for this project which used frozen epithelial cell aliquots that had been previously analysed for detection of HPV 16, 18, 31, 33 and 45.

According to the cytopathological diagnosis, the following groups were defined: normal (classification based

in a normal clinical, cytology and colposcopic examination), ASCUS (atypical squamous cells of undetermined significance), LSIL (low-grade intraepithelial lesion), HSIL (high-grade intraepithelial lesion) and invasive cervical carcinoma.

### Evaluation of HPV 16 and 18 DNA Integration

*Singleplex* real-time PCR with the SYBR<sup>®</sup> GreenER<sup>™</sup> (Invitrogen, California, USA) system with specific primers for E2 and E6 genes of HPV 16 and 18 were used as described by Huang *et al.*<sup>15</sup> A DNA physical status of HPV 16 and 18 was determined upon calculation of the ratio between E2 and E6 gene amplified products (E2/E6).

Viral DNA was considered to be in an episomal form when E2/E6 was 1 or higher and to be present in a linear form or in both episomal and linear forms, when E2/E6 was lower than 1.

### Persistent Infection Detection by mRNA Transcript Analysis

A NucliSENS EasyQ HPV<sup>®</sup> (bioMérieux S.A., Marcy l'Étoile, France) commercial system was used for the extraction, amplification and detection of E6/E7 mRNA transcripts of HPV 16, 18, 31, 33 and 45, according to the manufacturer's instructions. The E6/E7 mRNA amplification and detection consists in a NASBA reaction and qualitative mRNA transcript detection and result analysis was obtained by using a specific reader.

### HPV 16 e 18 viral DNA Quantification

The experimental procedure consisted in a real-time *duplex* PCR as described by Carcopino *et al.*<sup>11</sup> Taqman (Applied Biosystems; California, USA) specific primers and probes for E6 gene of HPV 16 and 18 as well as for exon 12 of the human albumin gene were used.

Viral load, expressed as the number of HPV copies per million of normal human cells (HPV cells /10<sup>6</sup> human cells) was calculated according to the following formula:

Viral load = Number of HPV copies/(Number of albumin copies/2) x 10.<sup>6</sup>

### Statistical Analysis

We used SPSS (Statistical Package for Social Sciences; SPSS, Inc., Chicago, USA) version 16.0 and chi-square ( $\chi^2$ ) test to analyse the association between molecular markers and development of intraepithelial lesions at a significance level of  $p = 0.05$ .

## RESULTS

### Assessment of HPV 16 and 18 DNA Integration

We analysed 65 patients, 44 with an HPV 16 infection and 21 with an HPV 18 infection.

According to cytological diagnosis, four groups were defined: normal ( $n = 26$ ), ASCUS ( $n = 16$ ), LSIL ( $n = 16$ ) and HSIL ( $n = 7$ ).

The HPV 16 DNA was in the episomal form in 23/44 (52.3%), in both linear and episomal form in 18/44 (40.9%)

and in the linear form in 3/44 (6.8%) of the patients (Fig. 1). According to cytological diagnosis, in 14/20 (70.0%) of normal cytologies, 3/11 (27.0%) of ASCUS and 6/10 (60.0%) of LSIL lesions, the HPV 16 DNA was in the episomal form. Concomitant forms were identified in 4/20 (20.0%) of normal cytologies, 8/11 (73.0%) of ASCUS cytologies, 4/10 (40.0%) of LSIL lesions and 2/3 (67.0%) of HSIL lesions, while the linear form was only detected in normal cytologies (2/20; 10.0%) and in premalignant lesions (1/3; 33.0%).

In the cases of HPV 18 infection, 4/21 (19.0%) presented the viral DNA in the episomal form, 6/21 (28.6%) in the concomitant form and 11/21 (52.4%) in the linear form (Fig. 2). According to cytological diagnosis, in 2/6 (33.3%) of normal cytologies, 1/5 (20.0%) of ASCUS and 1/6 (17.0%) of low-grade lesions, the HPV 18 DNA was in the episomal form. Both forms were identified in 2/6 (33.3%) of normal cytologies, 2/5 (40.0%) of the ASCUS, 1/6 (17.0%) of LSIL and 1/4 (25.0%) of HSIL lesions, while the linear form was detected in 2/6 (33.3%) of normal cytologies, 2/5 (40.0%) of ASCUS, 4/6 (67.0%) of LSIL and 3/4 (75.0%) of HSIL lesions.

No statistically significant association was identified between lesion grade severity and DNA physical state of HPV 16 ( $p = 0.108$ ) and HPV 18 ( $p = 0.783$ ).

**Detection of Persistent Infections using the mRNA Transcript Analysis**

We studied 299 patients with single and mixed infections by HPV 16, 18, 31, 33 and 45. Patients presenting an infection by two or more types of HPV were individually considered in the analysis ( $n = 349$ ). E6/E7 mRNA was detected in 67.6% (236/349) of the patients (Table 1). E6/E7 mRNA transcript over-expression was higher in HSIL lesions (83/102) and in invasive carcinoma (7/7). HPV

16 E6/E7 mRNA transcript over-expression was the most common in all cytological groups, followed by HPV 31 over-expression (8/77) in normal cytologies, HPV 33 (7/53) in ASC-US, HPV 45 (2/17) in ASC-H, HPV 18 (9/73) in LSIL lesions and HPV 31 (7/102) in HSIL lesions. In patients with an invasive cervical carcinoma only the HPV 16 (3/7), HPV 18 (3/7) and HPV 31 (1/7) E6/E7 mRNA were detected (Table 1).

In order to evaluate E6/E7 mRNA over-expression persistence, 61 patients were evaluated every six months over an 18-month period. From these, 7/61 (11.5%) presented a E6/E7 mRNA negative test at 6 to 18 months, meaning hr-HPV infection eradication. From the remaining 54 patients under clinical surveillance, 16 presented a negative E6/E7 mRNA test at 6 months after conization of premalignant lesions. Nine of the 38 patients, in whom E6/E7 oncogene over-expression persisted, underwent treatment and lesion progression was observed in 23 of the 29 patients who remained untreated.

**Quantification of HPV 16 and 18 viral DNA**

We assessed 131 patients with an HPV 16 infection, from which 84 were single infections. According to cytological diagnosis, five groups have been defined: normal ( $n = 46$ ), ASCUS ( $n = 24$ ), LSIL ( $n = 34$ ), HSIL ( $n = 25$ ) and cervical invasive carcinoma ( $n = 2$ ).

Regarding HPV 18, 60 patients were studied, from which 33 presented single infections, grouped according to cytological diagnosis in normal ( $n = 20$ ), ASCUS ( $n = 19$ ), LSIL ( $n = 13$ ), HSIL ( $n = 5$ ) and cervical invasive carcinoma ( $n = 3$ ).

The results obtained for HPV 16 allowed the identification of significant differences between viral DNA quantification and the severity of the lesion (Fig. 3).

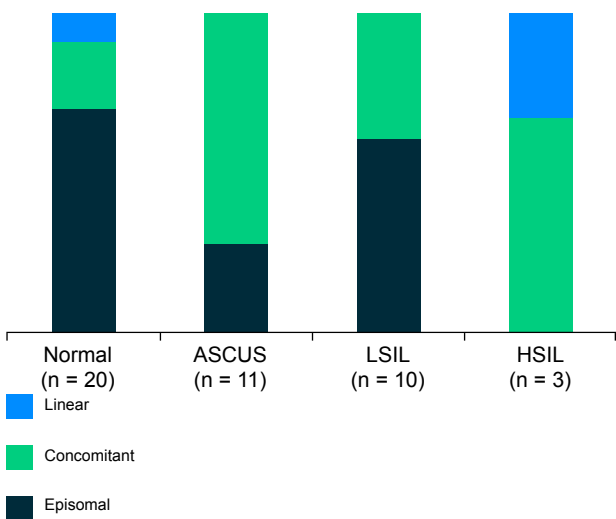


Figure 1 – Graphical representation of HPV 16 DNA physical status, according to cytological diagnosis (n = 44). Normal, normal cytology; ASCUS, undetermined significance atypia; LSIL, low-grade intraepithelial lesion; HSIL, high-grade intraepithelial lesion.

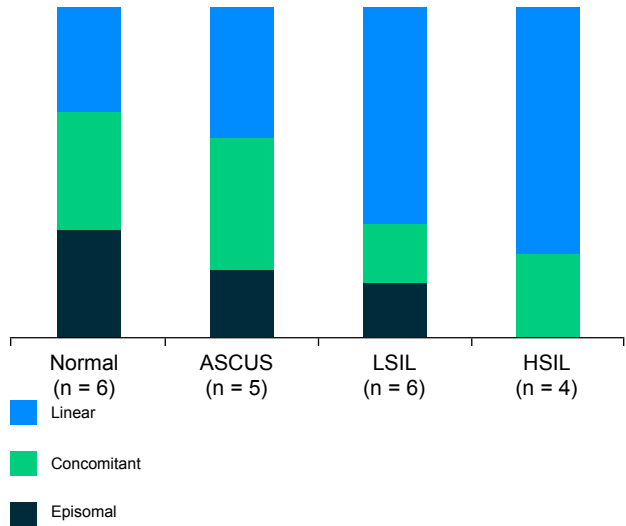


Figure 2 – Graphical representation of HPV 16 DNA physical status, according to cytological diagnosis (n = 21). Normal, normal cytology; ASCUS, undetermined significance atypia; LSIL, low-grade intraepithelial lesion; HSIL, high-grade intraepithelial lesion.

Table 1: Distribution of E6 and E7 oncogene transcripts over-expression, according to cytological diagnosis

<i>mRNA</i> (%)	Normal ( <i>n</i> = 77)	ASC-US ( <i>n</i> = 53)	ASC-H ( <i>n</i> = 17)	LSIL ( <i>n</i> = 73)	HSIL ( <i>n</i> = 102)	Carcinoma ( <i>n</i> = 7)	U/A ( <i>n</i> = 20)	Total ( <i>n</i> = 349)
<b>HPV 16 (<i>n</i> = 190)</b>								
-ve mRNA	14 (18.2)	5 (9.4)	2 (11.8)	11 (15.1)	7 (6,9)	0	3 (15.0)	42 (12.0)
+ve mRNA	23 (29.9)	19 (35.8)	8 (47.1)	26 (35.6)	61 (59,8)	3 (42.9)	8 (40.0)	148 (42.4)
<b>HPV 18 (<i>n</i> = 36)</b>								
-ve mRNA	5 (6.5)	2 (3.8)	0	2 (2.8)	1 (1,0)	0	1 (5.0)	11 (3.2)
+ve mRNA	3 (3.9)	4 (7.5)	1 (5.9)	9 (12.3)	5 (4,9)	3 (42.9)	0	25 (7.2)
<b>HPV 31 (<i>n</i> = 75)</b>								
-ve mRNA	15 (19.5)	6 (11.3)	2 (11.8)	10 (13.7)	7 (6,9)	0	3 (15.0)	43 (12.3)
+ve mRNA	8 (10.4)	5 (9.4)	0	7 (9.6)	7 (6,9)	1 (14.2)	4 (20.0)	32 (9.2)
<b>HPV 33 (<i>n</i> = 28)</b>								
-ve mRNA	3 (3.9)	2 (3.8)	1 (5.9)	1 (1.4)	3 (2,9)	0	1 (5.0)	11 (3.2)
+ve mRNA	0	7 (13.2)	1 (5.9)	4 (5.5)	5 (4,9)	0	0	17 (4.9)
<b>HPV 45 (<i>n</i> = 17)</b>								
-ve mRNA	2 (2.6)	2 (3.8)	0	1 (1.4)	1 (1,0)	0	0	6 (1.8)
+ve mRNA	4 (5.2)	1 (1.9)	2 (11.8)	2 (2.8)	5 (4,9)	0	0	14 (4.0)
<b>Total -ve mRNA</b>	<b>39 (50.6)</b>	<b>17 (32.1)</b>	<b>5 (29.4)</b>	<b>25 (34.2)</b>	<b>19 (18,6)</b>	<b>0</b>	<b>8 (40.0)</b>	<b>113 (32.4)</b>
<b>Total +ve mRNA</b>	<b>38 (49.4)</b>	<b>36 (67.9)</b>	<b>12 (70.6)</b>	<b>48 (65.8)</b>	<b>83 (81,4)</b>	<b>7 (100)</b>	<b>12 (60.0)</b>	<b>236 (67.6)</b>
<b><i>p</i> value</b>	<b>-</b>	<b>0.000</b>	<b>0.327</b>	<b>0.438</b>	<b>0.327</b>	<b>0.000</b>		<b>0.003</b>

Normal, normal cytology; ASC-US, undetermined significance atypia; ASC-H, undetermined significance atypia, not able to exclude a high-grade lesion; LSIL, low-grade intraepithelial lesion; HSIL, high-grade intraepithelial lesion; U/A, no information available; -ve mRNA, negative E6/E7 mRNA test; +ve mRNA, positive E6/E7 mRNA test.

In patients with a HPV 18 infection, according to cytological diagnosis, it was possible to observe that the viral load values were significantly increased according to the lesion grade, suggesting a similar relationship to the one identified for HPV 16 (Fig. 4).

HPV 16 and HPV 18 viral load average values were significantly higher in premalignant lesions than in low-grade lesions ( $p = 0.049$  and  $p = 0.001$ , respectively), particularly in patients between 31 and 40 years old. We also studied eight patients with concomitant HPV 16 and 18 infections and concluded that viral load determination had the same logarithmic order of magnitude for both genotypes.

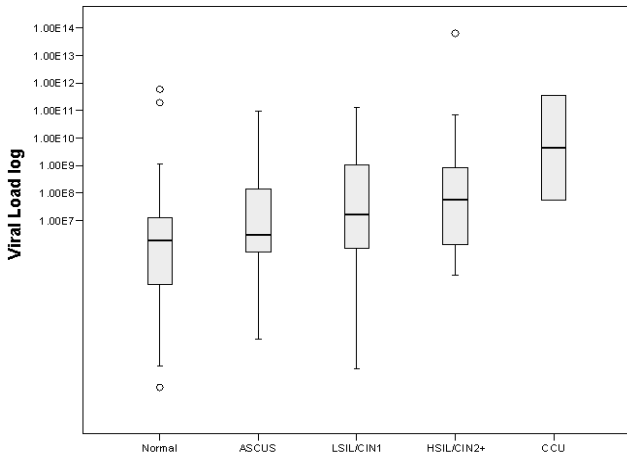
In patients who were followed-up upon premalignant lesions conization, a high viral load was detected for HPV 16 and 18 in 8/191 of the patients, suggesting the identification of persistent infections. In these patients, the viral load value remained unchanged or increased, in the same logarithmic order of magnitude. On the other hand, it was also possible to identify transient infections in 7/191 of the patients, confirmed by the absence of DNA viral load in the following 6 to 12 months.

## DISCUSSION

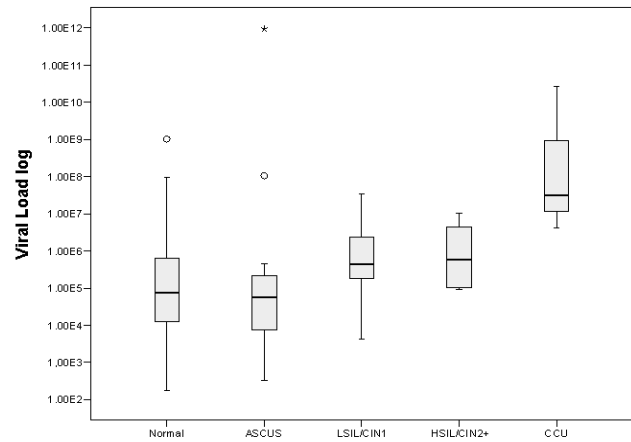
It is currently accepted that HPV DNA integration in the host cell genome is crucial for the malignant transformation process activating mechanisms for premalignant lesion progression.<sup>16</sup> However, the prognostic value of the study of the E2 gene integrity in cervical cancer development has been controversial.<sup>17</sup> There are studies that have identified the presence of episomal forms of HPV 16 DNA in cases of cervical cancer, suggesting that genome linearisation may not occur in every virus copy during the malignant transformation process.<sup>18,19</sup>

As regards HPV 18, studies have demonstrated that progression to premalignant lesions or cervical cancer may occur more readily when viral DNA is detected in its linear form.<sup>19</sup>

The results obtained in this study suggest that an HPV 16 DNA early integration event may in fact occur, as concomitant and linear forms were found in low-grade lesions and with normal cytology. The HPV 18 DNA physical state evaluation allowed for the identification of concomitant and linear forms in all cytological diagnostic groups. However, the presence of these forms increased in relation to the severity of cervical lesions. These results are



**Figure 3** – Graphic of HPV 16 viral load logarithm. Normal, normal cytology; ASCUS, undetermined significance atypia; LSIL/CIN1, low-grade intraepithelial lesion; HSIL/CIN2+, high-grade intraepithelial lesion; CCU, cervical cancer; °, outliers.



**Figure 4** – Graphic of HPV 18 viral load logarithm according to cytological diagnosis. Normal, normal cytology; ASCUS, undetermined significance atypia; LSIL/CIN1, low-grade intraepithelial lesion; HSIL/CIN2+, high-grade intraepithelial lesion; CCU, cervical cancer; ° \*, outliers.

in agreement with data described by other authors.<sup>20-22</sup>

The hr-HPV 16, 18, 31, 33 and 45 oncogenic potential is associated with E6/E7 oncogene activity and its interaction with cellular proteins p53 and pRB, leading to increased genomic instability and a risk of malignant transformation.<sup>23</sup> Therefore, the E6/E7 mRNA test, as it identifies the persistent over-expression of the viral oncogene transcripts, assumes a major role in the prognosis of low-grade lesions.<sup>24</sup> It is considered that the E6/E7 mRNA test presents a higher positive predictive value than DNA detection and typification methods, which only evaluate the persistence of a hr-HPV infection.<sup>24-26</sup>

E6/E7 mRNA was detected in 67.6% of the patients in our study. According to the cytological diagnosis, a statistical significant association between mRNA expression and lesion severity was observed ( $p = 0.003$ ; 81.4% in HSIL lesions *versus* 49.4% in normal cytologies) in agreement with other studies.<sup>24,25,27</sup>

The results of the follow-up study suggest that in patients with a cytological diagnosis of low-grade lesion, the detection of E6/E7 mRNA transcript over-expression may be considered as a major prognostic indicator, avoiding unnecessary treatments and improving patient's healthcare.<sup>25,28</sup>

In these situations, the E6/E7 mRNA test has a higher specificity for the early diagnosis of premalignant lesions, acting as a second-line test. E6/E7 oncogene over-expression persistence, observed in 15.8% of treated patients, suggests that it is efficient as a marker of lesion eradication, but not of infection eradication. However, the clinical usefulness of the E6/E7 mRNA test may be restricted as it only assesses oncogene over-expression in five types of hr-HPV.

It has been demonstrated that viral load determination

may be a good prognostic indicator for development of cervical cancer, as a high viral load may indicate active replication, originating viral persistence.<sup>29</sup> In our study, the values obtained in the normal cytology group have been the most heterogeneous, corresponding to the group where the highest value of viral load was observed. For this particular group, the analysis of the various parameters (cytological diagnosis, HPV infection and DNA quantification) may suggest the occurrence of a false negative result or a discrepancy with the cytological diagnosis. We may use the same rationale to explain an ASCUS lesion, in which HPV 16 DNA viral load was  $9.3 \times 10^{10}$ . Excluding these patients and taking into account the median values, we observed that the viral load increases with lesion grade, in agreement with general knowledge.<sup>9,11</sup>

According to the concept developed by Snidjers *et al.*,<sup>29</sup> our results demonstrate that progression may occur in 36/66 of the normal cytologies associated with HPV 16 infection (viral load  $> 10$ ).<sup>5</sup> In what concerns ASCUS lesions and results of viral DNA quantification, 21/43 of the lesions tend towards a reduction in lesion severity, with subsequent eradication of HPV 16 and/or 18 infection.

## CONCLUSIONS

This study suggests that HPV 16 and 18 DNA physical status determination is not on its own a good prognostic indicator of lesion development and progression. E6/E7 oncogene over-expression is associated with cervical lesions and presents a higher specificity for the early diagnosis of premalignant lesions. However, the predictive value of the E6/E7 mRNA test is variable according to the hr-HPV type. The hr-HPV DNA quantification seems to be one of the most promising prognostic indicators.

**CONFLICT OF INTERESTS**

The authors declare there are no conflict of interests regarding the writing of this manuscript.

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